REMARKS

Applicants have amended Claims 1, 4 and 11 to broaden their scope. Applicants have also added new Claims 21-23. Claims 2, 9 and 16 have been cancelled. In accordance with Ex parte Wirt, the total number of pending claims is not greater than the number of claims pending prior to the final Office Action. Ex parte Wirt, 1905 C.D. 247 (Comm'r Pat. 1905). Accordingly, Claims 1, 3-8, 10-15, and 17-23 are currently presented for examination.

Support for the amendments to Claims 1, 4, and 11 can be found at page 24, line 27 to page 26, line 11 and elsewhere in the specification. Support for new Claims 21-23 can be found, for example, in Table II. Accordingly, no new matter has been added to the application.

After careful review of the Office Action, Applicants respectfully traverse the Examiner's claim rejections as set out below.

Rejection of Claims Under 35 U.S.C. § 112, first paragraph - Written Description

The Examiner rejects Claims 1-4, 8-12 and 15-20 as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to a skilled artisan that Applicants had possession of the claimed invention at the time of filing the application. In particular, the Examiner asserts that Applicants have not described a sufficient number of specific compounds, other than antisense nucleic acids, that function to inhibit the activity or reduce the amount of a polypeptide comprising the amino acid sequence of SEQ ID NO: 325. Additionally, the Examiner asserts that Applicants have not described a sufficient number of proliferation-inhibitory antisense molecules targeted to genes having homology to SEQ ID NO: 165.

Applicants note the Examiner's acknowledgement that the present specification meets the written description requirement with respect to antisense nucleic acids which inhibit the activity or reduce the amount of a polypeptide comprising SEQ ID NO: 325 or which specifically target the gene comprising SEQ ID NO: 165. However, the Examiner asserts that the written description requirement is not satisfied with respect to Claims 1-4, 8-12 and 15-20 because, in addition to encompassing antisense nucleic acids, these claims encompass non-antisense

molecules which inhibit the activity or reduce the amount of a polypeptide comprising SEQ ID NO: 325.

Applicants note that Claim 1 and the claims dependent thereon recite a method of inhibiting cellular proliferation by inhibiting the activity or reducing the amount of a target polypeptide but do not include any elements reciting compounds. As stated in *Moba, B.V.* v. *Diamond Automation, Inc.*, "The test for compliance with §112 has always required sufficient information in the original disclosure to show that the inventor possessed the invention at the time of the original filing." *Moba, B.V. v. Diamond Automation, Inc.*, 325 F.3d 1306, 1320 (Fed. Cir. 2003). The present specification describes experiments demonstrating that inhibiting the activity or reducing the amount of a polypeptide comprising SEQ ID NO: 325 inhibits cellular proliferation. Applicants have also provided the sequences of antisense nucleic acids which inhibit proliferation through their effects on the polypeptide of SEQ ID NO: 325. The Examiner has acknowledged that the specification satisfies the written description requirement with respect to antisense nucleic acids which inhibit proliferation through their effects on the polypeptide of SEQ ID NO: 325. Applicants maintain that the description of the role of the polypeptide of SEQ ID NO: 325 in cellular proliferation coupled with the disclosure of agents which inhibit proliferation through their effects on the polypeptide constitutes sufficienct disclosure to permit those skilled in the art to recognize that Applicants were in possession of the claimed method at the time of filing. Accordingly, Applicants maintain that the written description requirement is satisfied with respect to Claim 1.

Independent Claims 4 and 11 and the claims dependent thereon recite methods of inhibiting cellular proliferation utilizing compounds. The claims encompass the use of antisense nucleic acids as well as other types of compounds which act on the recited targets which Applicants have identified as being involved in proliferation. The Examiner asserts that the written description requirement requires the disclosure of a sufficient number of compounds and that this requirement necessitates that Applicants provide the structures of compounds other than antisense nucleic acids which act on the recited targets.

In response, Applicants maintain that the written description requirement does not require the Applicants to provide the structure of every class of compound which acts on the recited target. Applicants have provided the sequence of the full-length *waa*E gene, and thus, the

complement thereof. Applicants have described methods for preparing specific fragments of this sequence such as transcription, chemical synthesis and enzymatic cleavage. Applicants have also provided detailed procedures for determining whether any fragment of the full-length antisense nucleic acid inhibits cellular proliferation by reducing the level or activity of a product of the *waa*E gene, such as the WaaE polypeptide of SEQ ID NO: 325. Applicants have validated their procedure for identifying inhibitory antisense by providing two specific examples of fragments of the full-length *waa*E antisense sequence which reduce the level or activity of the WaaE peptide thereby inhibiting proliferation. As acknowledged by the Examiner, by disclosing the sequence of the nucleic acid encoding the target polypeptide and a method for identifying additional proliferation-inhibiting antisense nucliec acids, Applicants have disclosed the structures of many antisense nucleic acids which inhibit the activity or reduce the amount of a polypeptide comprising SEQ ID NO: 325, thus providing the structures of a large number of molecules which fall within the scope of the claims.

In addition to the above disclosure of antisense nucleic acids Applicants describe numerous antisense nucleic-acid-like polymers that function to inhibit proliferation. For example, Applicants describe specific phosphodiester backbone replacement methods, for example, oligonucleotide synthesis using phosphorothioate nucleoside derivatives, which can be implemented with any of the antisense nucleic acids described in the specification, including the exemplary antisense nucleic acids of SEQ ID NOs: 459 and 460. (see page 102, line 26 to page 103, line 25). Antisense nucleic-acid-like polymers having phosphorothioate backbone replacements are known by those of skill in the art to have the same specificity and similar inhibitory effect as antisense molecules which contain a phosphodiester backbone (see, Lebedeva *et al.* at page 405 and Dove at page 123, cited in the Office Action mailed January 17, 2003). Additionally, the subject patent application describes and incorporates by reference the entire disclosure of U.S. Patent No. 5,142,047 ('047 patent), which teaches a method of creating a synthetic uncharged polynucleotide-binding polymer that corresponds to any phosphodiester antisense nucleic acid. The method can be used with any antisense nucleic acid, including those described and specifically exemplified in the subject patent application. The '047 patent also teaches that a synthetic uncharged polynucleotide-binding polymer possesses the same specificity and binding affinity for the target sequence as the antisense phosphodiester

oligonucleotide to which it corresponds. In view of these disclosures, Applicants maintain that they have described numerous compounds that reduce the amount or activity of a polypeptide comprising an amino acid sequence of SEQ ID NO: 325. Accordingly, Applicants maintain that the number of disclosed compounds is sufficient to satisfy the written description requirement with respect to the pending claims.

The Examiner also takes the position that the specification does not sufficiently describe antisense nucleic acids that inhibit the activity or reduce the amount of a product of a homolog of SEQ ID NO: 165. In particular, the Examiner contends that experimentation is required to determine whether a homolog is required for proliferation and that "one of skill in the art would not readily recognize the antisense molecules would inhibit proliferation." Applicants respectfully disagree.

Each of the claims have been amended to state that cellular proliferation is inhibited by inhibiting the activity or reducing the amount of either a proliferation-required *waa*E gene or a proliferation-required product of the *waa*E gene. As such, Applicants need only describe compounds that possess activity against homologs of *waa*E genes and *waa*E gene products that required for proliferation. Applicants submit that they have specifically described antisense nucleic acids which are complementary to at least a portion of a gene homologous to SEQ ID NO: 165 (see Table I, page 35 and Table VI, page 95) and which inhibit proliferation of the organisms containing those homologs. In particular, Applicants have demonstrated that the antisense nucleic acids of SEQ ID NOs: 459 and 460 inhibit the proliferation of three microorganisms having a proliferation-required gene that is 82% identical (*Klebsiella pneumoniae*), 82% identical (*Enterobacter cloacae*) and 81% identical (*Salmonella typhimurium*), to *E. coli waa*E (SEQ ID NO: 165). These homologous genes encode polypeptides that are 92% identical (*Klebsiella pneumoniae*), 94% identical (*Enterobacter cloacae*) and 93% identical (*Salmonella typhimurium*) to *E. coli* WaaE (SEQ ID NO: 325). Applicants maintain that the foregoing disclosure is sufficient to satisfy the written description requirement with respect to the pending claims.

With respect to whether experimentation is required to determine whether a homolog is required for proliferation, the present specification sets forth a method for determining whether a homolog is required for proliferation. In particular, the specification sets forth a technique for

generating antisense nucleic acids complementary to at least a portion of the homolog and for measuring the effect of the antisense nucleic acid on proliferation. (see pages 23, line 21 to page 26, line 10 and Examples 1-3) The disclosed technique can be implemented using standard methods of molecular biology. Accordingly, given the disclosure of the present specification one skilled in the art can readily determine whether a homolog is required for proliferation without undue experimentation.

With respect to the Examiners statements regarding the relevance of other issued U.S. Patents that claim a method of using a genus of compounds but which only describe one or a few compounds of that genus, Applicants recognize that each patent application is examined on its own merits with regard to the circumstances surrounding that application. The issued patents, which were identified in Applicants' response to the previous Office Action, were intended only to provide evidence thatthe U.S. Patent and Trademark Office regularly finds that the written description requirement is satisfied when the specification discloses one or a few compounds which act on a particular target which the Applicants have identified as being therapeutically important and the claims encompass the use of compounds which have the common attribute of acting on the recited target but which may have diverse structures.

In view of the above remarks, Applicants respectfully request that the Examiner withdraw his rejection of claims 1-4, 8-12 and 15-20 under 35 U.S.C. § 112, first paragraph.

Rejection of Claims Under 35 U.S.C. § 112, first paragraph - Enablement

The Examiner rejects Claims 1- 20 under 35 U.S.C. § 112, first paragraph because the specification allegedly does not enable a person skilled in the art to use the invention commensurate with the scope of the claims. In particular, the Examiner asserts that Applicants have not enabled the use of "antisense molecules as therapeutic compounds for treating subjects" because they do not teach methods of overcoming the alleged problems associated with premature degradation of the antisense molecule, delivery to the target cell and potential "by-stander" effects. Additionally, the Examiner asserts that it is not predictable whether antisense molecules that inhibit the activity or reduce the level of WaaE could inhibit proliferation in every cell type. Finally, the Examiner asserts that the specification does not enable one of ordinary

skill in the art to practice the claimed subject matter with compounds other than antisense nucleic acids.

With respect to enablement of the use of antisense compounds for the treatment of subjects (*in vivo*) use, Applicants maintain that the specification enables one skilled in the art to use the described antisense molecules successfully for *in vivo* therapy. Applicants have addressed the issue regarding the degradation of phosphodiester-based antisense molecules by describing phosphorothioate-based molecules having the same nitrogenous base composition and sequence as the phosphodiester-based sequences described therein. Synthesis of such phosphorothioate analogs is well within the knowledge of the art. Additionally, it is well known in the art antisense molecules having a phosphorothioate backbone are degradation resistant molecules that retain the inhibitory function of phosphodiester antisense molecule on which they are based. (see Lebedeva, page 405, cited in the Office Action mailed January 17, 2003). The specification also teaches PNA analogs of the phosphodiester-based sequences described therein (see page 103, line 14 to page 104, line 18). Synthesis of PNA analogs is well known in the art and such molecules are known to be highly resistant to degradation (see Soomets, et al., (1999) *Front Biosci*, **4**: D782-86, a copy of which is enclosed herewith as Exhibit A). Accordingly, given the teachings of the specification, one skilled in the art would be able to make and use several different degradation resistant antisense nucleic acids for *in vivo* therapeutic applications.

Applicants have also addressed the issue regarding delivery of the molecule to the target cell. In particular, the specification describes methods of administering and formulating antisense molecules for delivery to a target cell (see page 104, line 19 to page 106, line 6). Phosphodiesters and phosphorothioates both are internalized in the target cell without difficulty (see Lebedeva *et al.*, page 404). Although PNAs do not freely traverse the cell membrane, methods of delivering PNA to the interior of cells were known at the time of filing the subject application (see Soomets, *et al.*). As such, it was well within the ability of a skilled artisan to deliver phosphorothioate analogs and PNAs analogs of the specifically-described phosphodiester antisense molecules to target cells without undue experimentation.

With respect to the "by-stander" effect, Applicants respectfully submit that such effect does not influence whether one of ordinary skill in the art could practice the claimed invention without undue experimentation. Applicants note that the Examiner has not provided any

evidence that the antisense nucleic acids falling within the scope of the pending claims would actually result in "by-stander" effects. Instead the Examiner has only raised the theoretical possibility of such effects. Furthermore, if there were any "by-stander" effects on genes in the targeted cell other than those encoding the polypeptide of SEQ ID NO: 325 or comprising SEQ ID NO: 165, such effects would not be detrimental to the claimed methods because they would not be incompatible with the goal of inhibiting the proliferation of the targeted cell.

In addition, it is recognized in the art that single-gene accuracy is not essential for therapeutic applications of antisense molecules. (see, Branch, page 47, column 1, cited in the Office Action mailed January 17, 2003). Even if an antisense nucleic acid causes non-specific effects when administered to a subject, these non-specific effects do not necessarily prevent the claimed methods from being therapeutically valuable. Applicants again note that the Examiner has not provided any evidence that non-specific effects would result from the claimed methods but has only raised the theoretical possibility of such effects. Furthermore, even should there be "by-stander" effects with the antisense nucleic acids within the scope of the claims, Applicants note that many highly successful therapeutic molecules have unwanted side-effects. Furthermore, Applicants note that FDA approval is not a prerequisite for patentability. *In re Brana*, 51 F.3d 1560 (Fed. Cir. 1995).

With respect to the Examiner's contention that antisense therapy is an unpredictable field in general, Applicants respectfully disagree. Applicants note that in 2002, at least 9 antisense products were either on the market or in clinical trials (see Dove, page 122, cited in the Office Action mailed January 17, 2003). As of January 8, 2003, at least 77 antisense products were being tested at various stages of development (see Rajan, Antisense Products: Technology Markets, January 8, 2003, a copy of which is provided herewith as Exhibit B). Antisense technology is sufficiently predictable to fully enable the subject matter claimed in this patent application, even *in vivo* antisense therapy. Applicants need only provide sufficient disclosure to allow one of skill in the art practice the claimed subject matter without undue experimentation. At the time of filing the subject patent application, issues related to the stability, delivery and specificity of antisense molecules *in vivo* had been sufficiently resolved to allow one skilled in the art to practice the claimed subject matter without undue experimentation.

In view of the above remarks, Applicants respectfully submit that the subject patent application adequately teaches *in vivo* inhibition of cellular proliferation using antisense molecules described therein.

The Examiner also asserts that it is not predictable whether antisense molecules that inhibit the activity or reduce the level of WaaE could inhibit proliferation in every cell type. Although Applicants disagree with this assertion, to expedite prosecution, Applicants have amended the claims such that each claim requires that the compound that is used to inhibit cellular proliferation inhibits the activity or reduces the amount of a proliferation-required *waa*E gene or a proliferation-required product of the *waa*E gene. Thus, only homologs of the *E. coli waa*E gene or the *E. coli waa*E gene product which are required for proliferation are recited in the present claims. Additionally, the specification describes methods which allow one skilled in the art without undue experimentation to identify proliferation-inhibiting antisense molecules complementary to any proliferation-required homolog of the *waa*E gene (see Examples 1-3). Accordingly, Applicants maintain that a skilled artisan would be able to practice each of the claimed methods without undue experimentation.

Finally, the Examiner asserts that Applicants have not enabled a skilled artisan to practice the claimed subject matter with compounds other than antisense nucleic acids. Again Applicants respectfully disagree. The specification teaches numerous antisense compounds and antisense nucleic-acid-like polymers for use in the claimed methods. Furthermore, the specification teaches methods of identifying additional proliferation-inhibiting antisense nucleic acids complementary to at least a portion of the *waa*E gene or homologs of the *waa*E gene. Using methods taught in the specification, one skilled in the art can synthesize various polymers corresponding to these antisense nucleic acids which include, but are not limited to, phosphorothioate and PNA polymers. Such proliferation inhibiting antisense nucleic-acid-like polymers can be used in both *in vivo* and *in vitro* methods of inhibiting cellular proliferation as explained above.

In addition to the antisense nucleic-acid-like polymers, the specification teaches methods of screening for compounds, such as small molecules, which can be used in the claimed methods. The instant specification provides extensive guidance regarding the screening of both combinatorial chemical and natural product libraries, the construction of which is well known in

the art, to identify compounds that inhibit cellular proliferation by either inhibiting the activity or reducing the amount of a specific polypeptide, such as WaaE, or gene encoding such polypeptide. In particular, Example 8 describes both protein based assays, for identifying compounds that have activity against WaaE polypeptides, and cell based assays, which are use to identify compounds that have activity against either the *waa*E gene and/or its products (see Example 8, at pages 67, line 18 to 76, line 9). Example 9 provides a working example which verifies the effectiveness and specificity of the cell based assay for identifying compounds which inhibit proliferation by acting on a gene, such as *waa*E, or its gene products (see Example 9, at page 76, line 10 to page 79, line 26). Accordingly, Applicants have enabled one of ordinary skill in the art to practice the claimed methods using compounds other that antisense nucleic acids.

The Examiner is reminded that undue experimentation is not measured by the amount of time, expense or quantity of routine experimentation that is involved in implementing the disclosed methods. (see *In re Wands* 858 F.2d 731 (Fed. Cir. 1988); *United States v. Telectronics Inc.*, 857 F.2d 778 (Fed. Cir. 1998); and M.P.E.P. § 2164.06). Provided that procedure used to implement the claimed invention routine, it is of little consequence to enablement the number of iterations or the length of the procedure that is required before the end is achieved. As set out in Examples 1-3 and pages 102-106 (antisense molecules) as well as Examples 8-10 (small molecules and other compounds), Applicants have described in detail routine procedures for identifying proliferation-inhibiting compounds that are active against the *waa*E gene and products thereof. Accordingly, Applicants have enabled one of ordinary skill in the art to practice the subject matter of each of the claims presented for examination.

In view of the above remarks, Applicants respectfully request that the Examiner withdraw his rejection of claims 1-20 under 35 U.S.C. § 112, first paragraph.

CONCLUSION

Applicants believe that all outstanding issues in this case have been resolved and that the present claims are in condition for allowance. Nevertheless, if any undeveloped issues remain or if any issues require clarification, the Examiner is invited to contact the undersigned at the telephone number provided below in order to expedite the resolution of such issues.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: October 28, 2003

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ANTISENSE PROPERTIES OF PEPTIDE NUCLEIC ACIDS

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1. ABSTRACT

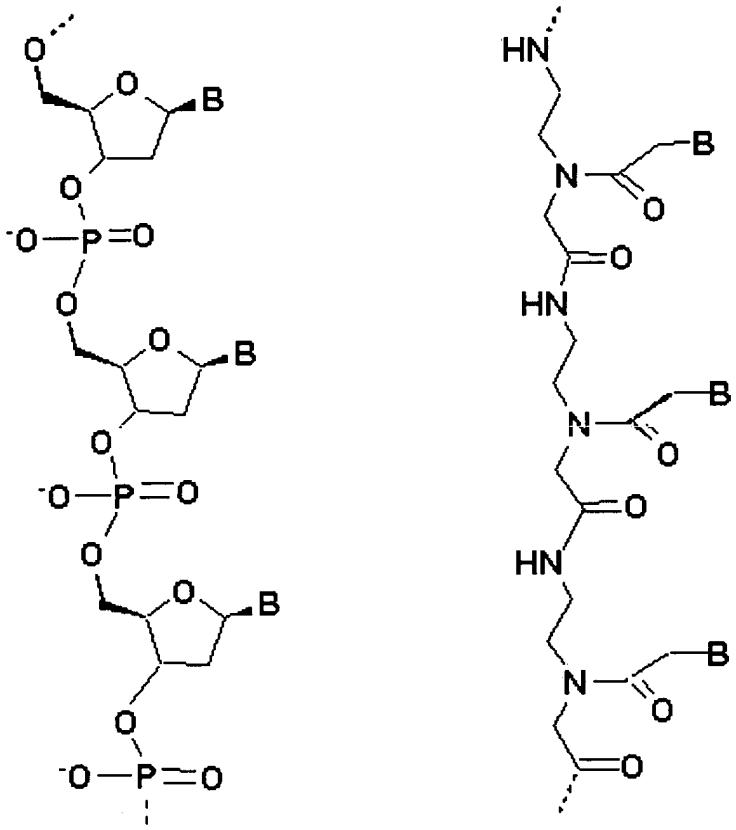
In this paper, a summary of recent antisense PNA (Peptide Nucleic Acid) applications is presented. We discuss in detail reports that demonstrate that the effects of PNA may be considerably improved by enhancing its cellular uptake.

2. INTRODUCTION

PNA is a nucleic acid analog with an achiral polyamide backbone consisting of N-(2-aminoethyl)glycine units (figure 1). The purine or pyrimidine bases are linked to the each unit via a methylene carbonyl linker (1-3) to target the complementary nucleic acid (4). PNA binds to complementary RNA or DNA in a parallel or antiparallel orientation following the Watson-Crick base-pairing rules (5-7). The uncharged nature of the PNA oligomers enhances the stability of the hybrid PNA/DNA(RNA) duplexes as compared to the natural homoduplexes. The non-natural character of the PNA makes PNA oligomers highly resistant to protease and nuclease attacks (8). These properties of PNA oligomers suggest that they could potentially serve as efficient antisense or antigene reagents. Indeed, peptide nucleic acids have been applied to block protein expression on the transcriptional (9) and translational level (10,11), and microinjected PNA oligomers demonstrate a strong antisense effect in intact cells (12). However, contrary to the "normal" nucleic acid analogs, PNA oligomers are not efficiently delivered into the cytoplasm of the cell, and until recently this has hindered the

Exhibit A

application of PNA oligomers as antisense reagents. In this work we summarize some recent achievements on PNA antisense application, especially these concerned with whole cell or tissue delivery of the PNA.



DNA **PNA**

3. PNAs AS ANTISENSE REAGENTS

This summary deals with two issues of PNA applications, the use of unmodified PNA as antisense reagent, and, the improvement of cellular uptake of PNA.

Although PNAs have several characteristics required for a good antisense molecule, they suffer from poor membrane penetrability. Therefore, the initial antisense experiments using PNA relayed on microinjection and cell permeabilization techniques.

Gray *et al* (13) have studied the uptake of different oligonucleotide analogues in transformed and non-transformed fibroblasts. In this study, PNA was shown to be poorly taken up by the cells, whereas transformed cells internalized all analogues better than non-transformed cells.The authors suggest that PNA and other uncharged oligonucleotide analogues are taken up through fluid-phase endocytosis, while charged analogues are internalized

through receptor mediated endocytosis

In 1992, the first study about the potential application of PNA was published (10). The microinjection into the nuclei of cultured cells of PNA complementary to the coding region of SV40 T antigen (T Ag) mRNA, caused down-regulation of translation of the respective protein. The extent of the inhibition was dose-dependent and specific when the intracellular concentration of PNA was below 5 μM. Moreover, the effect of the length of the PNA was investigated, using 10-, 15-, and 20-mer PNAs. The antisense effect was correlated to PNA length: at 1 μM concentration, the 20-, 15- and 10-mers gave 50%, 40% and 0% inhibition, respectively.

Bonham *et al* (12) published a study in which CV-1 cells, transiently transfected with T Ag expression vector plasmid, were microinjected with FITC-labeled PNA targeted against the T Ag. After injection they observed a quick redistribution of the fluorescent label into the nuclear compartment, whereas nucleoli were completely excluded. 6 h after injection, the expression of T Ag was inhibited by 57%. As a comparison, 10 times less of C-5 propynyl pyrimidine phosphorothioate oligonucleotides (propyne-S-ON), was needed for 70% inhibition. The authors suggest that the differences in inhibition are due to the capability of the propyne-S-ONs to activate RNAse H and due the lower rate of PNA association with the RNA than of the propyne-S-ON.

Several groups have used a rabbit reticulocyte cell-free translation system (10,11,14-17) or rat hepatocyte nuclear extract (10) to study antisense/antigene effects of PNAs *in vitro*. Using this technique, the inhibition of translation of the interleukin-2 receptor α subunit mRNA (10), chloramphenicol acetyltransferase mRNA (11), promyelocytic leukemia/retinoic acid receptor α mRNA (15,16) and inducible nitric oxide synthase mRNA (14) have been shown. In addition, the selective inhibition of mitochondrial DNA replication (18) and reverse transcription of the HIV-1 *gag* gene (17) by PNAs have been demonstrated. These results showed that the homopyrimidine or pyrimidine rich PNA sequences (as short as 6-mers) inhibited translation, whereas PNAs with mixed sequence had effect only when they were complementary to the AUG initiation codon region.

3.1. UNMODIFIED PNA

In 1998, the first *in vivo* results describing antisense effects of unmodified PNA were published by Tyler *et al* (19). In this study, 14-mer PNAs, directed against the neurotensin, NTR1, (position +103) and mu opioid (position -70) receptors mRNAs, were injected into the periaqueductal gray (PAG) of rats. Neurotensin as well as opioids are well known to exert an antinociceptive effect. In addition, neurotensin induces hypothermia. Behavioral studies of

anti-NTR1 or anti-opioid mu receptor PNA treated animals showed dramatically reduced responses to neurotensin and morphine, respectively. Furthermore, hypothermic effect of neurotensin was substantially reduced. These effects where reversible and responses returned to normal 5-14 days after final injection. The effects were specific since PNA targeted against the NTR-1 mRNA had no effect on the morphine response and the antisense PNA targeted to mu receptor mRNA did not decrease the effect of neurotensin. These changes were accompanied by a specific decrease of the number of neurotensin or morphine binding sites.

Continuing their work, Tyler *et al* (20) studied the effects of the antisense NTR1 PNA (injected intraperitoneally, i.p.) or sense NTR1 PNA (injected directly into the PAG of rats). Surprisingly, 24 h post injection, both sense and antisense PNAs decreased the antinociceptive effect of neurotensin injected into the PAG. However, mismatched anti NTR1 and mu opioid receptor PNAs showed no significant effect. A similar profile was found for the hypothermic responses of neurotensin. These effects were reversible and responses returned to normal 48 h post injection. The NTR1 mRNA level in brain was determined using quantitative PCR. The antisense PNA treated animals showed no change in NTR1 mRNA levels while the sense PNA treated animals showed a 50% decrease in mRNA levels, suggesting that the sense PNA acted by an antigene mechanism. In order to detect the uptake of PNA into the brain, a gel shift assay with brain homogenate was used. The data in (20) enable to evaluate the approximate concentration of the PNA in brain at 0.1-1 nM range. Such low and efficient concentration of the antisense PNA is at least remarkable.

The brain is protected from the surrounding blood vessels by a tight layer of non permeable cells, the blood-brain barrier (BBB), that restricts the access of many molecules, like glucose and amino acids into the brain. BBB protects the brain from infections and toxins, but also prevents the delivery of potential pharmacologically active molecules into the brain. Therefore, the results of Tyler *et al* (20) are surprising, especially since Pardridge *et al* (21) have shown that significant levels of unmodified PNA do not cross the BBB, and only the conjugation of the PNA to an anti-transferrin antibody allowed it to be transported through BBB. However, the concentration of PNA used by Tyler *et al* (10 mg/kg) was significantly higher than that was used in the other studies and the authors suggest that such a high amount of PNA may disrupt the BBB.

Aldrian-Herrada *et al* (22) have shown that PNAs can enter neurons in culture, probably through an endocytotic mechanism. Also, Taylor *et al* (18) have shown the uptake of PNAs by cultured human myoblasts and specific inhibition of replication of mutant mitochondrial DNA.

Good and Nielsen have recently published studies (23,24) showing that PNA can inhibit reporter gene expression in *E.coli*, probably through an antisense mechanism. The inhibition was shown to be specific and concentration dependent, and was more efficient in antibiotic permeable bacteria than in the wild type strain.

Some studies have shown anti-gene activity of PNAs in the cells showing its potential as possible regulator of gene expression. Vickers *et al* (25) studied the ability of 15-mer PNA to specifically block interaction of the transcription factor NF-kB with its binding site in the IL2-Rα promoter. Complete inhibition of transcription was shown when the cells were transfected with an IL2-Rα plasmid pre-incubated with PNA, while the treatment of cells with PNA after the transfection failed to modulate the transcription.

Boffa *et al* (26) reported that the 18-mer PNA complementary to the poly-CAG triplet area (27) of the androgen receptor and a TATA binding protein has a specific anti-gene activity in permeabilized prostatic cancer cells (28). Furthermore, the same authors showed that 17-mer PNA complementary to the sense strand of the second *myc* exon inhibits transcription in permeabilized COLO320-DM cells.

3.2. CARRIER MEDIATED UPTAKE OF PNA

Wittung *et al* have shown, using an liposomal model system for the plasma membrane, that PNAs do not readily diffuse through a membrane barrier (29). Furthermore, Bonham *et al* showed that incubating CV-1 cells with FITC-labeled PNA resulted only in cytoplasmic vesicular staining (12). Indeed, the neuron is the only cell type to date that has been shown to efficiently internalize unmodified PNAs, demonstrating the need for an effective transporter for these molecules for other tissues. To this end, several potential delivery systems have been developed.

Liposomal delivery that is often used for transfection with oligonucleotides has, to our knowledge, not been successfully used for PNA transport.

An interesting solution to the PNA uptake problem was proposed by Uhlmann *et al*. They showed that a synthetic PNA-DNA chimeric molecule was internalized by cells as efficiently as normal oligos (30) and, furthermore, in contrast to PNAs, were able to activate RNAse H (31).

Recent studies by several groups have shown that coupling of PNA to different carriers will improve their uptake into cells. Among these, several peptide sequences have been shown to be able to carry PNA oligomers across the cell membranes.

A short hydrophobic peptide with the sequence biotinyl-FLFL coupled to a PNA trimer has been shown to internalize into human erythrocytes and Namalwa cells (32). However, no data on transport of longer PNAs has been shown. Basu and Wickström (33) showed that PNA conjugated to an all-D-amino acid insulin-like growth factor 1 (IGF1) mimicking peptide was specifically taken up by cells expressing the IGF1 receptor, although no antisense activity was described.

In recent years, some peptides that translocate over the plasma membrane in an energy and endocytotic independent manner, have been designed and synthesized. An extensively studied sequence, derived from the third helix of the Antennapedia homeodomain (34), is called penetratin (for review cf. (35)). Penetratin or penetratin analogs have been used by us (36) and others (22,37) to transport PNAs over the plasma membrane of cells in culture. Moreover, we used the chimeric peptide transportan as an alternative transport peptide, showing that penetratin is not the only transport peptide that can mediate PNA transport (36). The conjugation of a transporter peptide to PNA greatly improved uptake in neurons (22) and was necessary for any significant uptake in Bowes (36) and DU-145 cells (37).

In their study (22) Aldrian-Herrada and co-workers synthesized antisense PNA against the starting codon region of prepro-oxytocin mRNA. Treatment of cultured magnocellular oxytocin neurons with antisense PNA or vector peptide-PNA conjugate resulted in reduced immunohistochemical signal for prepro-oxytocin and reduced amount of oxytocin mRNA in a dose- and time-dependent manner. The mechanism behind the decrease of mRNA levels is not clear yet, but the authors suggest that the PNA-induced RNA degradation could occur in RNaseH independent metabolic pathways.

In our work (36), we synthesized 21-mers antisense PNA complementary to positions 1-21 and 18-38 of the coding region of human galanin receptor type 1 (hGalR1) mRNA. Since unmodified PNA uptake by Bowes melanoma cells was not significant we used the cell penetrating constructs where PNA was conjugated to the penetratin or transportan via a disulfide bridge. After the treatment of cells with antisense constructs the inhibition of 125I-galanin binding was measured and the maximal effect was obtained with a construct targeting area 18-38 in hGalR1 mRNA. The PNA constructs were more potent than phosphorothioates and phosphodiester oligomers designed to target the same area in the coding region of hGalR1 mRNA. The down-regulation of hGalR1 level was dose-dependent and specific since no effect was observed after the treatment of cells with scrambled PNA construct.

In addition we used the PNA complementary to region 18-38 of the rat galanin receptor type 1 in vivo experiments. The intrathecal

administration of antisense construct into the spinal cord of rats reduced the effect of intrathecal galanin approximately 100-fold. Again, the effect was specific since treatment with scrambled PNA construct did not affect the galanin inhibition of the flexor reflex.

4. PERSPECTIVES

It seems that the period in antisense research described as the "end of the beginning" (Crooke), fairly describes the situation in PNA application as well. The stability and high affinity of PNA oligomers have been demonstrated as well as their in vitro applicability. The recent achievements in PNA transport have fueled the interest in *in vivo* application of PNA, and, the first results in this field are promising.

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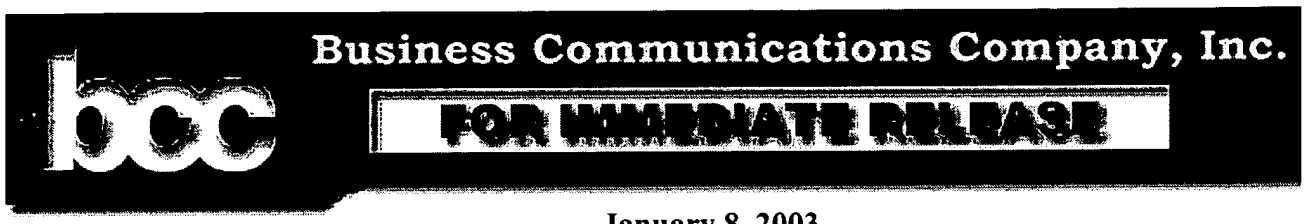

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POTENTIAL MARKET FOR ANTISENSE THERAPEUTICS IN 2007 TO CROSS $3.15 BILLION

The use of antisense compounds as therapeutics is beginning to gain momentum. After several years of developing the technologies needed to succeed, antisense therapeutics are gaining a reputation as therapeutics with excellent safety profiles that are well-tolerated by patients and easy to administer. This was shown in 1998 when the first antisense therapeutic, Vitravene™, was approved for treating CMV retinitis. Vitravene™ gave creditability to all antisense therapeutics and launched them into the competitive world of therapeutics development.

According to a soon-to-be-released report from **Business Communications Company, Inc.** (www.bccresearch.com) ***RB-170 Antisense Products: Technology, Markets,*** the potential market for antisense therapeutics in 2007 is estimated to be over $3.15 billion or 3.1% of the combined total market for all applications of antisense therapeutics.

The adaptability of antisense to develop therapeutics to treat any gene expression related disorder is illustrated by the wide variety of applications in which antisense therapeutics are currently being developed, including cancer, coronary heart disease, type 2 diabetes, immunological disorders (asthma, Crohn's disease, ulcerative colitis, multiple sclerosis, psoriasis, and rheumatoid arthritis), infectious diseases (CMV retinitis, HBV, HCV, HIV, and HPV), polycystic kidney disease, and retinal disorders (age-related macular degeneration, diabetic retinopathy, and proliferative vitreoretinopathy). Antisense therapeutics are being developed by 18 companies covered in this report. There are currently 42 antisense therapeutics in development, resulting in 77 preclinical studies, clinical trials, or a product on the market. All but one antisense therapeutic are either in the preclinical or clinical development stages.

The potential 2007 market for antisense therapeutics for treatment of immunological disorders is the largest, with a potential value of nearly $1.8 billion, followed by cancer at $760 million, infectious disease at $310 million, and all other applications at over $330 million.

The companies that will become leaders of the antisense industry must not only develop successful therapeutics, but must also extend the use of antisense technology. Chemical modifications have produced a variety of modes in the administration of antisense therapeutics, as well as increased stability. Antisense technology is also being used to address other areas, such as therapeutics

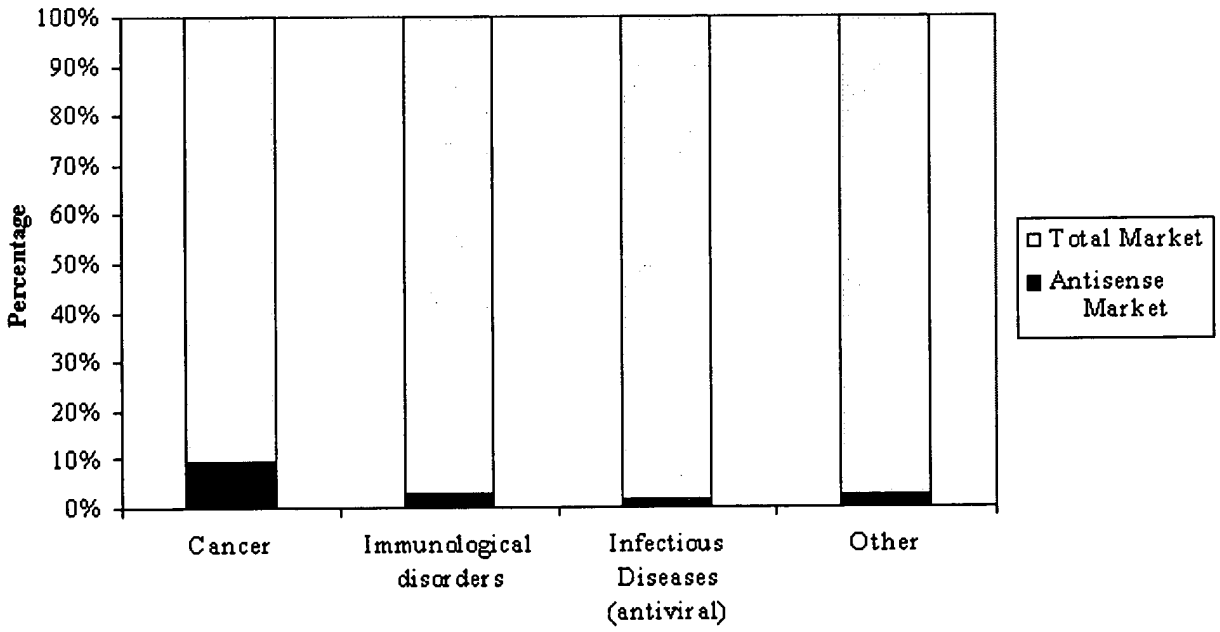
Exhibit B

discovery. One of the major bottlenecks of therapeutics development is the research and discovery process. New techniques are being developed using antisense compounds for gene function analysis, target validation, and discovery of new therapeutics. Antisense compounds are hoped to provide a much-needed tool that can be used in the high throughput screening method of therapeutic agents.

Potential Market for Antisense Therapeutics in 2007
($ Billions)

	Antisense Market	Total Applications Market	% of Total Market
Cancer	0.76	7.39	10.3
Immunological disorders	1.75	63.50	2.8
Infectious Diseases (antiviral)	0.31	18.30	1.7
Other	0.33	13.40	2.5
Total	3.15	102.59	3.1

Potential Market for Antisense Therapeutics in 2007
($ Billions)



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